

00/22/00

TO 23 MAR 2000

PCT



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REQUEST FOR FILING NATIONAL PHASE OF
PCT APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

To: Asst. Commissioner of Patents
 and Trademarks
 Washington, D.C. 20231

(Our Deposit Account No. 03-3975)

TRANSMITTAL LETTER TO THE UNITED STATES
 DESIGNATED/ELECTED OFFICE (DO/EO/US)

Atty Dkt: PM 266291 /PHM.7025/UST
M# /Client Ref.

From: Pillsbury Madison & Sutro LLP, IP Group:

Date: March 23, 2000

This is a **REQUEST** for **FILING** a PCT/USA National Phase Application based on:

1. International Application <u>PCT/GB98/02820</u> <u>↑ country code</u>	2. International Filing Date 17 September 1998 Day MONTH Year	3. Earliest Priority Date Claimed 23 September 1997 Day MONTH Year (use item 2 if no earlier priority)
--	---	---

4. Measured from the earliest priority date in item 3, this PCT/USA National Phase Application Request is being filed within:

(a) ☐ 20 months from above item 3 date (b) ☒ 30 months from above item 3 date,

(c) Therefore, the due date (unextendable) is March 23, 2000

Title of Invention ANTISENSE OLIGONUCLEOTIDES AGAINST THYMIDYLATE SYNTHASE

6. Inventor(s) KOROPATNICK, Donald James et al

Applicant herewith submits the following under 35 U.S.C. 371 to effect filing:

☒ Please immediately start national examination procedures (35 U.S.C. 371 (f)).

☒ **A copy of the International Application** as filed (35 U.S.C. 371(c)(2)) is transmitted herewith (file if in English but, if in foreign language, file only if not transmitted to PTO by the International Bureau) including:

- a. ☒ Request;
- b. ☒ Abstract;
- c. 29 pgs. Spec. and Claims;
- d. 13 sheet(s) Drawing which are ☒ informal ☐ formal of size ☒ A4 ☐ 11"

9. ☒ **A copy of the International Application has been transmitted by the International Bureau.**

10. **A translation of the International Application** into English (35 U.S.C. 371(c)(2))

- a. ☐ is transmitted herewith including: (1) ☐ Request; (2) ☐ Abstract;
 (3) _____ pgs. Spec. and Claims;
 (4) _____ sheet(s) Drawing which are:
☐ informal ☐ formal of size ☐ A4 ☐ 11"
- b. ☐ is not required, as the application was filed in English.
- c. ☐ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
- d. ☐ Translation verification attached (not required now).

RE: USA National Filing of PCT/GB98/02820

11. ☒ **PLEASE AMEND** the specification before its first line by inserting as a separate paragraph:
a. ☒ --This application is the national phase of international application PCT/GB98/02820 filed September 17, 1998 which designated the U.S.--
b. ☐ --This application also claims the benefit of U.S. Provisional Application No. 60/_____, filed _____.--
12. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., **before 18th month** from first priority date above in item 3, are transmitted herewith (file only if in English) including:
13. ☒ PCT Article 19 claim amendments (if any) have been transmitted by the International Bureau
14. ☐ Translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., of **claim amendments** made before 18th month, is attached (required by 20th month from the date in item 3 if box 4(a) above is X'd, or 30th month if box 4(b) is X'd, or else amendments will be considered canceled).
15. **A declaration of the inventor** (35 U.S.C. 371(c)(4))
a. ☐ is submitted herewith ☐ Original ☐ Facsimile/Copy
b. ☒ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
16. **An International Search Report (ISR):**
a. Was prepared by ☒ European Patent Office ☐ Japanese Patent Office ☐ Other
b. ☒ has been transmitted by the international Bureau to PTO.
c. ☒ copy herewith (2 pg(s).) ☒ plus Annex of family members (2 pg(s).).
17. **International Preliminary Examination Report (IPER):**
a. ☒ has been transmitted (if this letter is filed after 28 months from date in item 3) in English by the International Bureau with Annexes (if any) in original language.
b. ☒ copy herewith in English.
c.1 ☐ IPER Annex(es) in original language ("Annexes" are amendments made to claims/spec/drawings during Examination) including attached amended:
c.2 ☐ Specification/claim pages #____ claims #
Dwg Sheets #
d. ☐ Translation of Annex(es) to IPER (required by 30th month due date, or else annexed amendments will be considered canceled).
18. **Information Disclosure Statement** including:
a. ☒ Attached Form PTO-1449 listing documents
b. ☐ Attached copies of documents listed on Form PTO-1449
c. ☒ A concise explanation of relevance of ISR references is given in the ISR.
19. ☐ **Assignment** document and Cover Sheet for recording are attached. Please mail the recorded assignment document back to the person whose signature, name and address appear at the end of this letter.
20. ☐ Copy of Power to IA agent.
21. ☐ **Drawings** (complete only if 8d or 10a(4) not completed): ____ sheet(s) per set: ☐ 1 set informal;
☐ Formal of size ☐ A4 ☐ 11"
22. ☐ ____ (No.) **Verified Statement(s)** establishing "small entity" status under Rules 9 & 27
23. **Priority** is hereby claimed under 35 U.S.C. 119/365 based on the priority claim and the certified copy, both filed in the International Application during the international stage based on the filing in (country) GREAT BRITAIN of:
- | | <u>Application No.</u> | <u>Filing Date</u> | | <u>Application No.</u> | <u>Filing Date</u> |
|-----|------------------------|-----------------------|-----|------------------------|----------------------|
| (1) | <u>9720107.3</u> | <u>Sept. 23, 1997</u> | (2) | <u>9722012.3</u> | <u>Oct. 17, 1997</u> |
| (3) | <u>9812140.3</u> | <u>June 6, 1998</u> | (4) | _____ | _____ |
| (5) | _____ | _____ | (6) | _____ | _____ |
- a. ☒ See Form PCT/IB/304 sent to US/DO with copy of priority documents. If copy has not been received, please proceed promptly to obtain same from the IB.
b. ☐ Copy of Form PCT/IB/304 attached.

24. Attached: 5 pages of Sequence Listing

25. Preliminary Amendment:

25.5 Per Item 17.c2, cancel original pages #_____, claims #_____, Drawing Sheets #26. **Calculation of the U.S. National Fee (35 U.S.C. 371 (c)(1)) and other fees is as follows:**Based on amended claim(s) per above item(s) ☐ 12, ☐ 14, ☐ 17, ☐ 25, ☐ 25.5 (hilitte)

Total Effective Claims	minus 20 =	x \$18/\$9	= \$0	966/967
Independent Claims	minus 3 =	x \$78/\$39	= \$0	964/965
If any proper (ignore improper) Multiple Dependent claim is present,		add \$260/\$130	+0	968/969

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): →→ BASIC FEE REQUIRED, NOW →→→→

A. If country code letters in item 1 are not "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

See item 16 re:

1. Search Report was <u>not prepared</u> by EPO or JPO -----	add \$970/\$485		960/961
2. Search Report was prepared by EPO or JPO -----	add \$840/\$420	+840	970/971

SKIP B, C, D AND E UNLESS country code letters in item 1 are "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

→ <input type="checkbox"/> B. If <u>USPTO</u> did not issue <u>both</u> International Search Report (ISR) and (if box 4(b) above is X'd) the International Examination Report (IPER), -----	add \$970/\$485	+0	960/961
→ <input type="checkbox"/> C. If <u>USPTO</u> issued ISR but not IPER (or box 4(a) above is X'd), -----	add \$690/\$345	+0	958/959
→ <input type="checkbox"/> D. If <u>USPTO</u> issued IPER but IPER Sec. V boxes <u>not all</u> 3 YES, -----	add \$670/\$335	+0	956/957
→ <input type="checkbox"/> E. If international preliminary examination fee was paid to <u>USPTO</u> and Rules 492(a)(4) and 496(b) <u>satisfied</u> (IPER Sec. V <u>all</u> 3 boxes YES for <u>all</u> claims), -----	add \$96/\$48	+0	962/963

27. SUBTOTAL = \$840

28. If Assignment box 19 above is X'd, add Assignment Recording fee of ----\$40 +0 (581)

29. Attached is a check to cover the ----- TOTAL FEES \$840

Our Deposit Account No. 03-3975

Our Order No. 9901

266291

C#

M#

CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 and 492 (missing or insufficient fee only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown above for which purpose a duplicate copy of this sheet is attached.

This CHARGE STATEMENT does not authorize charge of the issue fee until/unless an issue fee transmittal form is filedPillsbury Madison & Sutro LLP
Intellectual Property Group1100 New York Avenue, NW
Ninth Floor
Washington, DC 20005-3918
Tel: (202) 861-3000
Atty/Sec: DJB/mhn

By Atty: Donald J. Bird

Sig:

Reg. No. 25323

Fax: (202) 822-0944
Tel: (202) 861-3027NOTE: File in duplicate with 2 postcard receipts (PAT-103) & attachments.

ANTISENSE OLIGONUCLEOTIDES AGAINST THYMIDYLATE SYNTHASE

This invention relates to antisense oligodeoxynucleotides targeted to sequences in thymidylate synthase (TS) mRNA. In particular the invention relates to antisense oligodeoxynucleotides targeted to sequences in the 3' end of TS mRNA, which antisense oligodeoxynucleotides are both cytostatic on their own when administered to human tumour cell lines, and also enhance the toxicity of the anticancer drugs such as Tomudex administered to those cells. In contrast, antisense oligodeoxynucleotides targeted to sequences at or near the translation start site at the 5' end of TS mRNA have either no effect, or enhance cell growth, when administered on their own. In addition, antisense nucleic acids targeted to these 5' sequences (but not to 3' sequences) induce TS gene transcription. The invention also relates to a combination product comprising an antisense oligodeoxynucleotide in combination with an anticancer agent such as Tomudex (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid) or the Zeneca development compound ZD 9331 ((S)-2-(2-fluoro-4-[N-(4-hydroxy-2,7-dimethylquinazolin-6-ylmethyl)-N-(prop-2-ynyl)amino]benzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid), and to the use of such a combination product in the treatment of cancer.

Thymidylate synthase (TS) (EC 2.1.1.45) catalyses the conversion of deoxyuridylate to thymidylate, and is a housekeeping enzyme essential for the only intracellular *de novo* synthesis of thymidylate (Danenbergs, 1977). TS gene expression is tightly regulated with respect to cell proliferation state (Maley and Maley, 1960; Lochsin *et al.*, 1979). As such, the TS gene is part of a group of genes whose expression is elevated at the G₁/S cell cycle boundary, and it has been suggested that transcription of several S-phase genes (including dihydrofolate reductase and thymidine kinase) is controlled in part by the E2F family of transcription factors (Farnham *et al.*, 1993; Mudrak *et al.*, 1994). In fact, transfection of active E2F1 genes into mouse cells induces expression of TS and other S-phase and cell cycle-regulated genes (De Gregori *et al.*, 1995). As cells progress through the cell cycle from G₀ through S phase, TS mRNA levels increase approximately 20-fold and TS enzyme activity increases about 10-fold (Navalgund *et al.*, 1980). However, TS gene transcription rate is upregulated only 2 to 4 times, suggesting that post-transcriptional events play a major role in TS regulation (Ayusawa *et al.*, 1986; Jenh *et al.*, 1985; Johnson, 1994). Differences in TS

mRNA stability are not likely to be critical in regulation, as TS mRNA half-life is about 8 hours in both resting and growing rodent cells (Jenh *et al.*, 1985). On the other hand, TS mRNA translation appears to be regulated by the TS protein itself, which specifically interacts at two sites within its own mRNA to inhibit protein production (Chu *et al.*, 1991, 1993b, 1994; Voeller *et al.*, 1995). Translation of other mRNAs (including *c-myc* mRNA) may also be regulated by interactions with TS protein (Chu *et al.*, 1995).

Because of its role in DNA precursor synthesis, TS has been identified as a potential target for cancer chemotherapeutic agents (Hardy *et al.*, 1987). High TS levels have been correlated with poor prognosis in patients with ovarian cancer (Suzuki *et al.*, 1994), rectal cancer (Johnston *et al.*, 1994) childhood acute non-lymphoblastic leukaemia (Volm *et al.*, 1994), and non-small cell lung carcinoma (Volm and Mattern, 1992). However, its prognostic value is not high in all tumour types (Peters *et al.*, 1986, 1994). Two types of TS inhibitors have been developed: (a) nucleotide analogues (including 5-FU, its riboside, and deoxyriboside derivatives) which must be activated to 5-fluorodeoxyuridylate (FdUMP) within cells to be effective (Heidelberger *et al.*, 1983) and (b) 5,10-CH₂FH₄ (antifolate) analogues, including *N*-10-propargyl-5,8-dideazafolate (CB3717) (Calvert *et al.*, 1986) and Tomudex (ZD1694; *N*-[5-(*N*-[3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl]-*N*-methylamino)-2-thenoyl]-L-glutamic acid) (Jackman *et al.*, 1991a, 1991b). Although Tomudex and 5-FU inhibit TS and have potent cytotoxic and antitumour activity (Heidelberger *et al.*, 1983; Keyomarsi *et al.*, 1993), they have an unusual biochemical effect. When human cancer cell lines are treated with 5-FU or Tomudex, TS levels increase rapidly, perhaps as a result of the release of translational inhibition by the TS protein (Keyomarsi *et al.*, 1993; Chu *et al.*, 1990; Chu *et al.*, 1993a).

It has been speculated that the release of translational inhibition that accompanies binding and inactivation of TS by chemotherapeutic agents (including Tomudex and 5-FU) might be prevented by treating cells with agents that could replace the specific interaction between TS mRNA and TS protein, and inhibit translation (Keyomarsi *et al.*, 1993) but no such agents were described. In another speculative article it was hypothesised that antisense nucleic acids designed to both reduce the ability of TS mRNA to direct protein production, and to interact with the TS protein binding site, may be useful in complementing the effectiveness of drugs targeted against TS (Rapaport *et al.*, 1992).

In previous patent applications, UK 9720107.3 and UK 9722012.3, we disclosed how to specifically down-regulate the expression of TS in human breast cancer (MCF-7) cells in two ways. First, we both transiently and stably transfected the cells with vectors expressing antisense RNA molecules directed to hybridise to three different regions of the TS mRNA.

5 Targeted sequences were: (1) sequences participating in the formation of a putative stem-loop structure surrounding the translation start site, and immediately adjacent and 3' to that site (these sequences also participate in binding TS protein to modulate translation), (2) the exon1/exon2 boundary and (3) the 3' end of the mature cytoplasmic mRNA. Antisense TS RNA was expressed from these vectors (as assessed by northern blot analysis and a novel
10 modification of the run-on transcription assay to measure antisense transcription against background constitutive TS gene expression) (Koropatnick *et al.*, 1997). Second, we transiently transfected cells with single-stranded oligodeoxynucleotides targeted to hybridise to: (a) the translation start site and sequences surrounding it, (b) a sequence proximal to the translation start site and participating in the putative stem-loop structure, and (c) the
15 translation stop site near the 3' end of the mature cytoplasmic RNA.

The present invention is based on our discovery that an antisense oligonucleotide, oligo 83, complementary to a sequence in the TS mRNA 3' untranslated region, down-regulated the level of TS mRNA and protein, inhibited cell proliferation and enhanced the cytotoxicity of TS-directed chemotherapy drugs.

20 In a first aspect of the invention we provide an antisense oligodeoxynucleotide which hybridises to a target nucleic acid sequence in thymidylate synthase and which selectively inhibits thymidylate synthase production in mammalian cells. Preferably the oligonucleotide is targeted to sequences at or near the translational stop site at the 3' end of the TS gene, which sequences lie in the region between bases 800 and 1600, using the sequence numbering
25 described for human thymidylate synthase mRNA by Takeishi *et al.*, 1985. More preferably the sequences lie in the region between bases 1000 and 1530. Most preferably the sequences lie in the region between bases 1030 and 1460.

In a second aspect of the invention we provide an antisense oligodeoxynucleotide which hybridises to a target nucleic acid sequence in thymidylate synthase and which
30 selectively enhances thymidylate synthase production in mammalian cells. Preferably the oligonucleotide is targeted to sequences at or near the translation start site at the 5' end of the

TS gene, which sequences lie in the region between bases 1 and 300, using the sequence numbering in Takeishi *et al.*, 1985. More preferably the sequences lie in the region between bases 50 and 200. Most preferably the sequences lie in the region between bases 90 and 130.

An antisense oligodeoxynucleotide is an oligonucleotide which is designed to
5 hybridise to a specific region of a target nucleic acid sequence. The target nucleic acid is the TS gene or mRNA transcribed from the TS gene. Preferably the target nucleic acid is the mRNA encoding thymidylate synthase.

The effects of antisense oligonucleotides on thymidylate synthase expression can be measured using procedures which are well known to persons skilled in the art. In the present
10 application, effects on mRNA levels have been measured by Northern blot analysis and nuclear run-on transcription assay, and effects on the growth of human tumour cells have been measured by counting cell numbers using a Coulter counter.

Antisense oligonucleotides to thymidylate synthase may inhibit, stimulate or have no effect on thymidylate synthase expression. Of these, preferred antisense oligonucleotides are
15 those which either inhibit or stimulate thymidylate synthase expression, and particularly preferred antisense oligonucleotides are those which inhibit thymidylate synthase expression.

By inhibition of thymidylate synthase expression we mean inhibition of at least 10% relative to the untreated control, measured at day 4 using the assay described in Example 1.2. Preferably inhibition of thymidylate synthase expression is at least 20% and most preferably
20 inhibition is at least 40%.

By stimulation of thymidylate synthase expression we mean stimulation of at least 10% relative to the untreated control, when measured at day 7 using the assay described in Example 1.2. Preferably stimulation is at least 20% and most preferably stimulation is at least
25 40%.

Preferably, the antisense oligonucleotides are from about 8 to about 50 nucleotides in length, more preferably from about 12 to about 40 nucleotides in length and most preferably from about 16 to about 30 nucleotides in length.

Specific examples of sequences of antisense oligonucleotides which regulate thymidylate synthase activity are shown in Table 1. The regions of TS mRNA targeted by the
30 oligonucleotides are shown in Figure 7.

Table 1:

ANTISENSE OLIGONUCLEOTIDE	SEQUENCE
OLIGO 83	TTAAGGATGTTGCCACTGGC
OLIGO 86	AATGGCTGTTTAGGGTGCTT
OLIGO 90	TGTGGCCGGCTCGGAGCTGC
OLIGO 91	GCGCCATGCCTGTGGCCGGC
OLIGO 92	GCGCCATGCCTGTGGCCGGC
OLIGO 93	CCCGCCCGCCGCGCCATGCC
ODN 32	ATGCGCCAACGGTTCCTAAA
PAS/TSS	UGUGGCCGGCUCGGAGCUGCCGCGCCGGCC
PAS/EXON1,2	GCUACAGCCUGAGAGAUGAAUUCCCUCUGC

It will be appreciated that the invention is not restricted merely to those specific antisense oligonucleotides which are disclosed in Table 1 above but encompasses
 5 oligonucleotides of from about 8 to about 50 nucleotides in length which selectively inhibit or selectively enhance thymidylate synthase production and which are selected from those regions of the TS gene which are described hereinbefore.

Hybridisation of an antisense oligonucleotide to its target nucleic acid sequence is mediated by the formation of hydrogen bonds between complementary bases on each nucleic
 10 acid strand. Hybridisation may occur between nucleic acid strands which have varying degrees of complementarity, depending on the hybridisation conditions employed. The term "specifically hybridisable" is used to describe an oligonucleotide which has a sufficient degree of complementarity to ensure stable, specific binding to its target sequence, whilst avoiding non-specific binding to non-target sequences.

15 Antisense oligonucleotides may be designed to hybridise to any region within the thymidylate synthase mRNA molecule, including the coding region, the 5'untranslated region, the 3'untranslated region, the 5'cap region, introns and intron/exon splice junctions.

Hybridisation of the antisense oligonucleotide to thymidylate synthase mRNA may affect any aspect of mRNA function, for example mRNA translocation, mRNA splicing,
 20 mRNA translation, or the feedback inhibition mechanism regulated by the binding of

thymidylate synthase protein to binding sites within the thymidylate synthase mRNA molecule.

An oligonucleotide is a polymeric molecule which is assembled from nucleotide or nucleoside monomers. The monomers may consist of naturally occurring bases, sugars and inter-sugar linkages or may also contain non-naturally occurring derivatives which modify the properties of the oligonucleotide, for example, phosphorothiorated oligonucleotides have been used in the present application to increase resistance to nuclease degradation.

Preferred oligonucleotides may contain phosphorothiorates, phosphotriesters, methyl phosphonates or short chain alkyl, cycloalkyl or heteroatomic intersugar linkages. Other preferred oligonucleotides may be methoxy-ethoxy winged or may contain a peptide nucleic acid backbone. Particularly preferred oligonucleotides are those containing phosphorothiorates (Summerton, J.E. and Weller, D.D., U.S. Patent No: 5,034,506).

The oligonucleotides may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "Protocols for Oligonucleotides and Analogues; Synthesis and Properties," Methods in Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition.

In a further aspect of the invention, there is provided a pharmaceutical composition comprising an antisense oligonucleotide targeted to thymidylate synthase as defined hereinbefore in a pharmaceutically acceptable diluent or carrier.

In a further aspect of the invention there is provided a method for the treatment of cancer (or a method for providing an antiproliferative effect) which comprises administering to a warm-blooded animal an effective amount of an oligonucleotide targeted to thymidylate synthase as defined hereinbefore. The invention also provides the use of such an oligonucleotide in the production of a new medicament for the treatment of cancer (or for the treatment of proliferative disease.

In a further aspect of the present invention, there is provided a combination product comprising an antisense oligonucleotide targeted to thymidylate synthase in combination with an anticancer agent. The antisense oligonucleotide and the anticancer agent may be administered separately, sequentially, simultaneously or in a mixture.

The anticancer agent may cover three main categories of therapeutic agent:

(i) thymidylate synthase inhibitors such as Tomudex (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid) (European Patent Application no. 0239362, Example 7, compound no. 8 therein); Zeneca development compound ZD9331 ((S)-2-(2-fluoro-4-[N-(4-hydroxy-2,7-dimethylquinazolin-6-ylmethyl)-N-(prop-2-ynyl)amino]benzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid) (European Patent Application no. 0562734, Example 3 thereof); LY 231514 (Eli Lilly Research Labs, Indianapolis, IN); 1843U89 (Glaxo-Wellcome, Research Triangle Park, NC); AG337 and AG331 (both by Agouron, La Jolla, CA) (Touroutoglou and Pazdur, Clin. Cancer Res., 2, 227-243, 1996).

(ii) cytostatic agents such as antioestrogens (for example tamoxifen, toremifene, raloxifene, droloxifene, idoxifene), progestogens (for example megestrol acetate), aromatase inhibitors (for example anastrozole, letrozole, vorazole, exemestane), antiprogestogens, antiandrogens (for example flutamide, nilutamide, bicalutamide, cyproterone acetate), LHRH agonists and antagonists (for example goserelin acetate, luprolide), inhibitors of testosterone 5 α -dihydroreductase (for example finasteride), anti-invasion agents (for example metalloproteinase inhibitors like marimastat and inhibitors of urokinase plasminogen activator receptor function) and inhibitors of growth factor function, (such growth factors include for example EGF, FGFs, platelet derived growth factor and hepatocyte growth factor such inhibitors include growth factor antibodies, growth factor receptor antibodies, tyrosine kinase inhibitors and serine/threonine kinase inhibitors).

(iii) antiproliferative/antineoplastic drugs and combinations thereof, as used in medical oncology, such as antimetabolites (for example antifolates like methotrexate, fluoropyrimidines like 5-fluorouracil, FUdR, florafur, FdUR, purine and adenosine analogues, cytosine arabinoside); antitumour antibiotics (for example anthracyclines like doxorubicin, daunomycin, epirubicin and idarubicin); mitomycin-C, dactinomycin, mithramycin; platinum derivatives (for example cisplatin, carboplatin, oxaliplatin); alkylating agents (for example nitrogen mustard, melphalan, chlorambucil, busulphan, cyclophosphamide, ifosfamide, nitrosoureas, thiotepa); antimitotic agents (for example vinca alkaloids like vincristine and taxoids like taxol,

taxotere); topoisomerase inhibitors (for example epipodophyllotoxins like etoposide and teniposide, amsacrine, topotecan).

The anticancer treatment may also be radiotherapy.

5

Particularly preferred anticancer agents are thymidylate synthase inhibitors such as Tomudex (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid) (European Patent Application no. 0239362, Example 7, compound no. 8 therein) and the Zeneca development compound ZD9331 ((S)-2-(2-fluoro-4-[N-(4-hydroxy-2,7-dimethylquinazolin-6-ylmethyl)-N-(prop-2-ynyl)amino]benzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid) (European Patent Application no. 0562734, Example 3 thereof).

In a further aspect of the present invention there is provided a pharmaceutical composition comprising a combination product as defined hereinbefore and a pharmaceutically acceptable diluent or carrier.

Any pharmaceutical composition as defined hereinbefore may be in a form suitable for oral use, for example a tablet, capsule, aqueous or oily solution, suspension or emulsion; for topical use, for example a cream, ointment, gel or aqueous or oily solution or suspension; for nasal use, for example a snuff, nasal spray or nasal drops; for vaginal or rectal use, for example a suppository; for administration by inhalation, for example as a finely divided powder such as a dry powder, a microcrystalline form or a liquid aerosol; for sub-lingual or buccal use, for example a tablet or capsule; or particularly for parenteral use (including intravenous, subcutaneous, intramuscular, intravascular or infusion), for example a sterile aqueous or oily solution or suspension. In general the above compositions may be prepared in a conventional manner using conventional excipients.

Tomudex is conveniently administered to humans by intravenous injection of a sterile aqueous solution at a dose in the range, for example, of 1 to 4 mg/m² of body surface area once every three weeks, preferably at a dose of 3 mg/m² once every three weeks.

ZD 9331 is conveniently dosed to humans by oral administration of a solid dosage form or by intravenous injection of a sterile aqueous solution. The oral dosage form is conveniently administered to humans at a total dose in the range, for example, of about 1 to

100 mg/kg (i.e. about 35 mg/m² to 3.5 g/m²) every three weeks given by a continuous or an intermittent dosing schedule, for example a dosing schedule of a three week dosing cycle comprising daily doses on days 1 to 5 only followed by no further doses until the next dosing cycle or a dosing schedule of a four week dosing cycle comprising daily doses on days 1 to 14 only followed by no further doses until the next dosing cycle. Preferably the oral dosage form is administered to humans at a total dose in the range, for example, of about 1 to 30 mg/kg every three or four week dosing cycle. The sterile aqueous solution is conveniently administered intravenously to humans at a total dose of up to 100 mg/m² every three weeks given by a continuous or an intermittent dosing schedule, for example, a dosing schedule of one dose per three week dosing cycle, a dosing schedule of a three week dosing cycle comprising daily doses on days 1 to 5 only followed by no further doses until the next dosing cycle, a dosing schedule of a three week dosing cycle comprising doses on days 1 and 8 only followed by no further doses until the next dosing cycle or a dosing schedule of a three week dosing cycle comprising continuous infusion on days 1 to 5 followed by no further dosing until the next dosing cycle. Preferably the sterile aqueous solution is administered intravenously to humans at a total dose in the range, for example, of about 20 to 50 mg/m² every three weeks given by a continuous or an intermittent dosing schedule as illustrated hereinbefore.

The antisense oligonucleotide is conveniently administered to humans by intravenous injection of a sterile aqueous solution at a dose per dosing cycle in the range, for example, of 0.1 µg to 1g, preferably at a dose of 1mg to 100mg.

The amount of active ingredient that is combined with one or more excipients to produce appropriate dosage forms will necessarily vary depending upon the particular component of the combination product, the host treated and the particular route of administration. For example, a formulation intended for oral administration to humans will generally contain, for example, from 0.5 µg to 2g of active agent compounded with appropriate and convenient amounts of excipients which may vary from about 5 to about 98 percent by weight of the total composition. A formulation intended for parenteral administration to humans will generally contain 0.1 µg to 50mg. Dosage unit forms will generally contain about 1 µg to about 500mg of an active ingredient.

In a further aspect of the invention there is provided a method for the treatment of cancer (or a method for providing an antiproliferative effect) which comprises administering to a warm-blooded animal an effective amount of a combination product as defined above. The invention also provides the use of such a combination product in the production of a new medicament for the treatment of cancer (or for the treatment of proliferative disease).

Abbreviations used in this application are set out below.

TS	thymidylate synthase
CMV	cytomegalovirus
5-FU	5-fluorouracil
LFA	lipofectamine
PBS	phosphate-buffered saline
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
FBS	fetal bovine serum
MT	metallothionein
ODN	oligodeoxynucleotide
bp	base pairs
DMEM	Dulbecco's modified Eagle medium
oligo	oligonucleotide

The invention will now be illustrated but not limited by reference to the following Example and Figures wherein:

Figure 1 shows that MCF-7 cell growth is inhibited by transfection with antisense TS oligo 86 (targeted to the translation stop site), but is enhanced by transfection with antisense TS oligos 90 or 92 (targeted to sequences at or near the translation start site).

Cells were transiently-transfected with 0.5 or 1.0 μ M antisense TS oligonucleotides in Lipofectin as described. Cell numbers were measured by Coulter counter in triplicate flasks after 4 days of growth. Control cells were treated with Lipofectin without oligonucleotides. Cell growth is expressed as a percentage of growth of control cells.

*: Significantly higher than control ($p < 0.05$, one way analysis of variants).

**: Significantly lower than control ($p < 0.05$, one way analysis of variants).

Figure 2 shows that HeLa cell growth is inhibited by transfection with antisense TS oligo 86 (targeted to the translation stop site), but is enhanced after transfection with antisense TS oligo 91 (targeted to the translation start site).

HeLa cells were transfected with 0.05 or 0.10 μM antisense TS oligonucleotides in Lipofectin (10 $\mu\text{g/ml}$) for 4 hours as described. Note that oligo concentrations are considerably lower than those used for MCF-7 cells. The efficiency of Lipofectin-mediated DNA transfection of HeLa cells is greater than for MCF-7 cells. Lipofectin was removed, the cells were trypsinised, and 25,000 viable cells per flask were plated in tissue culture flasks. Cell numbers were measured by Coulter counter in triplicate flasks after 4, 7 and 8 days of growth. Control cells were treated with Lipofectin without oligonucleotides. Cell growth is expressed as a percentage of growth of control cells.

*: Significantly higher than control ($p < 0.05$, Student's t-test).

**: Significantly lower than control (Student's t-test).

Figure 3 shows that HeLa cell growth is inhibited by transfection with antisense TS oligo 83 (targeted to a 3' untranslated sequence downstream of the translation stop site), but is not affected by transfection with antisense TS oligo 81 (targeted to the translation start site). The experimental protocol was as described in the legend to Figure 2.

Figure 4 shows that transient transfection of HeLa cells with oligo 86 (targeted to the TS translation stop site) enhances sensitivity to Tomudex and that oligo 91 (targeted to the TS translation start site) reduces sensitivity to Tomudex.

HeLa cells were transfected with 0.05 and 0.10 μM antisense TS oligonucleotides and plated in flasks at low density, as described for Figure 2. Tomudex (0-8 nM) was added (triplicate flasks for each Tomudex concentration) and the cells allowed to proliferate for 7 days. Cell numbers were measured by Coulter counting at that time. Survival is plotted as a percentage of growth in cells transfected with oligonucleotide, but untreated with Tomudex. Therefore, these data reveal inhibition or enhancement of Tomudex killing independent of

growth inhibition or enhancement induced by oligonucleotides in the absence of Tomudex. The mean of three values is plotted. Error bars were smaller than the size of the symbol in each case.

*: Significantly different from control ($p < 0.05$, Student's t-test).

Figure 5 shows that transient transfection of HeLa cells with oligo 83 (targeted to a sequence in the 3' untranslated region of TS mRNA) enhances sensitivity to Tomudex whereas oligo 81 (targeted to a 3' sequence downstream of that targeted by oligo 81) has no effect on Tomudex sensitivity.

HeLa cells were transfected with 0.10 μM antisense TS oligonucleotides and plated in flasks at low density, as described for Figure 2. Tomudex (0-10 nM) was added (triplicate flasks for each Tomudex concentration) and the cells allowed to proliferate for 4 days. Cell numbers were measured by Coulter counting at that time. Survival is plotted as a percentage of growth in cells transfected with oligonucleotide, but untreated with Tomudex. Therefore, these data reveal enhancement of Tomudex killing independent of growth inhibition induced by oligonucleotides in the absence of Tomudex. The mean of three values is plotted. Error bars were smaller than the size of the symbol in each case.

*: Significantly different from control ($p < 0.05$, Student's t-test).

Figure 6 shows that antisense TS oligo 91, but not oligo 86, stimulates TS gene transcription in human HeLa cells.

The same HeLa cells for which data are presented in Figure 2 were assessed for run-on transcription of TS, glyceraldehyde phosphate dehydrogenase (GAPDH), and 18S rRNA genes. Briefly, cells were transfected with 0.05 and 0.10 μM antisense TS oligonucleotides in Lipofectin (10 $\mu\text{g/ml}$), or with Lipofectin alone (LFA control) for 4 hours as described. Lipofectin was removed and cells were trypsinised and replated in tissue culture flasks. Four days after transfection, nuclei were isolated from approximately 5×10^6 cells for each treatment and initiated TS, GAPDH, and 18S rRNA transcripts allowed to incorporate [^{32}P]-CTP for 30 minutes. Alcohol-precipitable radiolabeled RNA was hybridised for 48 hours to unlabeled TS, GAPDH, and 18S rRNA cDNA immobilised in triplicate dots on nylon membrane as described. Relative transcription rate is presented as:

$$\text{Relative transcription rate} = \frac{\text{(hybridisation signal from gene of interest)}}{\text{(hybridisation signal for GAPDH or 18S rRNA genes)}}$$

Figure 7 shows the sequence of human mRNA for thymidylate synthase (EC 2.1.1.45), bases 1 to 1536.

Figure 8: shows that antisense TS ODN 83 inhibits HeLa cell proliferation. HeLa cells transfected with 50 nM antisense TS ODN 83 (●) or 50 nM scrambled control ODN 32 (○) were assessed for cell proliferation at 1, 2, 5, and 6 days following transfection. Data points indicate the average of two measurements, and are representative of qualitatively similar results obtained in 16 independent experiments.

Figure 9 shows that antisense TS ODN 83 suppresses HeLa cell growth after transfection, followed by recovery to control proliferation rate after 48 hours. HeLa cells were transfected with 50 nM antisense TS ODN 83 or 50 nM scrambled control ODN 32 as described in the legend to Figure 1. Values derived from cells transfected with ODN 32 were normalised to 100%, and each bar indicates the percent of that value measured following treatment with ODN 83 (mean \pm SE of 4 independent experiments). Asterisks (*) indicate significant differences ($p < 0.02$, Student *t*-test).

Figures 10 & 11 show that treatment of HeLa cells with ODN 83 leads to decreased TS mRNA levels.

(Figure 10) HeLa cells were transfected with ODN 83 or scrambled control ODN 32, or treated with Lipofectamine alone. Cells were harvested at 1, 2, and 4 days post-transfection and total cellular RNA isolated, reverse-transcribed, and TS and GAPDH cDNA amplified by 24 PCR cycles in the same reaction vessel. TS (208 bp) and GAPDH (752 bp) RT/PCR products were confirmed by Southern blotting and hybridisation to specific radioactively-labeled probes.

(Figure 11) TS:GAPDH ratio of RT/PCR products from RNA isolated from HeLa cells 1 day

after transfection with ODN 83 or ODN 32. Twenty-four, 25, 26, or 27 cycles of PCR amplification were carried out, revealing the same reduction in TS:GAPDH ratio after transfection with ODN 83.

5 Figure 12 shows that TS protein levels (inferred by measurements of 5-FdUMP binding) are diminished by antisense TS ODN 83 but not scrambled control ODN 32. 5-FdUMP binding was measured in cells transfected with ODN 83 (hatched bars) or ODN 32 (open bars) at different times following transfection.

(A): Results are plotted as a percent of 5-FdUMP binding in cells transfected with control
10 ODN 32 \pm SE ($n=5$). The values for ODN 32 ($n=5$) were normalised to 100% and are shown without error bars.

(B): Results are presented as pmol 5-FdUMP bound per mg total protein ($\times 10^{-3}$) to reveal that transfection with control ODN 32 had no significant effect on TS protein levels. Error bars indicate errors calculated according to a Student *t*-test, and indicate error due to differences in
15 experimental conditions in 5 measurements taken on different days, and differences due to transfection with different ODNs. The asterisks indicate significant differences ($p<0.02$) determined by using a paired Student *t*-test.

20 Figure 13: shows that antisense TS ODN 83 sensitises HeLa cells to the toxic effects of 5-FU, 5-FUdR, Tomudex, and MTX, but not cisplatin or chlorambucil. HeLa cells were transfected with ODN 83 (●) or control ODN 32 (○) and treated with different concentrations of 5-FU (A), 5-FUdR (B), Tomudex (C), MTX (D), cisplatin (E) or chlorambucil (F) for 4 days, beginning 24 h after transfection. Data points are plotted as the mean \pm SE of 4 measurements. Where error bars are not apparent, they are obscured by the symbol. Asterisks
25 (*) indicate significant differences <0.02 , Student *t*-test).

Example 1

Example 1.1: Experimental Methods

Cell culture:

MCF-7 (human breast adenocarcinoma) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 10 mM Hepes (pH 7.4) and 0.1% Gentamycin.

Vector construction:

Expression vectors pAS/TSS and pAS/exon1,2 were designed to produce, upon transfection into MCF-7 cells, single-stranded antisense RNA molecules containing double-stranded 30 bp oligonucleotides complementary to the TS mRNA at one of two sites.

Oligodeoxynucleotides corresponding to each strand of the human TS cDNA at positions 111 to 140 (pAS/TSS; targeting a 30 bp region adjacent to, and 2 bp away from, the translation start site) or 296 to 325 (pAS/exon1,2; targeting a 30 bp region spanning the exon 1/exon 2 boundary) were synthesized. Numbering of bases was according to GenBank accession no. X02308 (Takeishi *et al.*, 1985). In order to facilitate cloning, six additional nucleotides were incorporated (5 at the 5' end, 1 at the 3' end) of each oligodeoxynucleotide to produce *Hind* III or *Xba* I sticky ends when complementary strands were annealed. Single-stranded oligonucleotides (4 mg each) were annealed in 3X SET (450 mM NaCl, 60 mM Tris-HCl, 3 mM EDTA, pH 7.8) by treating the mixture for 5 min at 90°C, 5 h at 50°C, then 16 h at 25°C. Double-stranded products were identified by gel electrophoresis, and directionally inserted into the *Hind* III and *Xba* I sites of pRC/CMV (Invitrogen Corp., San Diego, CA). The orientation of cloning was confirmed by direct sequencing.

Oligodeoxynucleotides:

Fully phosphorothioated 20-mer oligonucleotides (ODNs) were synthesized by Isis Pharmaceuticals (Carlsbad, CA). The 6 terminal nucleotides at the 5' and 3' ends of each ODN were 2'-methoxyethoxy-modified to make them resistant to intracellular nucleases and increase

their stability within cells (McKay *et al.*, 1996), but the internal 8 nucleotides without methoxyethoxy groups were susceptible to RNase H cleavage. Oligo 86 was complementary to TS mRNA from base positions 1035 to 1054 (GenBank accession no. X02308; Takeishi *et al.*, 1985), which surround the TS mRNA translation stop site (UAG at bases 1045 to 1047). Oligo 90 was complementary to base positions 111 to 130, which are 3' and proximal to the translation start site (AUG at bases 106 to 109). Oligo 92 was complementary to base positions 101 to 120, including and surrounding the translation start site.

Transfection:

Antisense RNA expression vectors were transfected into MCF-7 cells using Lipofectamine (GIBCO BRL, Burlington, ON, Canada), a polycationic liposome formulation. 1.5×10^6 cells were dispensed into 100 X 15 mm tissue culture plates and allowed to adhere overnight. Cells were washed once with 3 ml OptiMEM (GIBCO BRL, Burlington, ON, Canada), followed by exposure to 6 ml of a mixture of double-stranded expression vector DNA (0.83 $\mu\text{g/ml}$) plus Lipofectamine (1.67 $\mu\text{g/ml}$) in opti-MEM for 6 h at 37°C in a 5% CO₂ incubator. The DNA/Lipofectamine mixture was removed and replaced with 10 ml DMEM with supplements. Control cells were transfected with pRC/CMV vector without insert. Transiently-transfected cells were used within 1 to 6 days. Stably-transfected cells were allowed to recover in complete non-selective medium without Geneticin for 48 h, then grown in the presence of Geneticin (400 μg per ml, active form [Gibco/BRL]) to allow selection of colonies harboring pRC/CMV control vectors, or pRC/CMV vectors expressing RNA complementary to sequences surrounding the translation start site (pAS/TSS).

Single-stranded oligodeoxynucleotides (0.5 μM or 1.0 μM) were transiently transfected into 1.5×10^6 MCF-7 cells adhering to 100 X 15 mm tissue culture plates, in a total volume of 5 ml in the presence of Lipofectamine (2 $\mu\text{g/ml}$) in Opti-MEM. Control cells were treated with

Lipofectamine/Opti-MEM without added DNA. Cells were washed after 6 h, medium plus supplements added as described above, and then grown for 48 hours before isolation of nuclei for run-on transcription measurement.

Southern blot analysis:

09509418-07100
007720-27460560

Total DNA was isolated from pAS/TSS transfected cells as follows. Cells were incubated in lysis buffer (50 mM Tris-HCl [pH8.0], 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate, 0.5 mg/ml proteinase K) for 6 h at 55°C. One-third the volume of 6 M NaCl was added to precipitate non-nucleic acids by centrifugation at 10,000 X g for 15 min. The DNA in the supernatant was precipitated in isopropanol and washed with 70% ethanol. DNA was cleaved with *Hind* III for 16 h and analysed by Southern blotting (Sambrook *et al.*, 1989). Blots were hybridized with an [α -³²P]dCTP random primer-labeled pAS/TSS probe (Church and Gilbert, 1984), and were exposed to a phosphor screen and quantitated using a PhosphorImager and the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). The nylon membranes were stripped and rehybridized with an *Alu* probe (300 bp of a human *Alu* restriction fragment inserted into pBR322 [Jelinek *et al.*, 1980]) in order to quantitate the amount of human DNA loaded in each lane (Koropatnick *et al.*, 1988).

Northern blot analysis:

RNA was isolated using RNeasy columns (Qiagen Inc., Chatsworth, CA) from cells transfected with the pAS/TSS expression vector. Ten or 15 ug of RNA per lane were separated on a 1.4% formaldehyde gel (Sambrook *et al.*, 1989) and transferred to a Hybond-N nylon membrane. Membranes were hybridized (Church and Gilbert, 1984) with either a pAS/TSS-generated riboprobe (Promega Corp., Madison, WI) designed to bind to antisense RNA, or a random primer-labeled, 1.9 kb *Xho* I fragment from pcHTS-1 (a eukaryotic expression vector containing the human TS cDNA: a generous gift from Dr. K. Takeishi, University of Shizuoka, Shizuoka, Japan)(Takeishi *et al.*, 1985). Blots were stripped and rehybridized with cDNA probes to detect 18S ribosomal RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Images were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Isolation of nuclei:

Relative transcription rates were determined by a nuclear run-on assay (Koropatnick *et al.*, 1997), a modification of the methods of Kikuchi *et al.* (1992) and Almendral *et al.* (1988). Nascent transcripts were extended *in vitro* (Marzluff and Huang, 1984) in parallel reactions using MCF-7 cell nuclei isolated 48 h following transient transfection with control or antisense TS RNA expression vectors, or single-stranded antisense TS oligodeoxynucleotides (or

Lipofectamine alone), and from cells stably transfected with antisense RNA expression vectors. Adherent cells were rinsed twice with ice-cold PBS, scraped off with a rubber policeman, pelleted in PBS (5 min, 500 X g) and lysed by incubating 5 min at 4° C in 4 ml of lysis buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40). All subsequent steps were carried out at 4° C. Complete cell lysis and integrity of released nuclei was checked by light microscopy, and nuclei were pelleted by centrifugation at 500Xg for 5 min. Nuclei were then resuspended in 4 ml of lysis buffer by vortexing, pelleted by centrifugation, resuspended in 200 µl of nuclei storage buffer (40% glycerol, 5 mM MgCl₂, 50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) in a 15 ml conical polypropylene centrifuge tube, and immediately frozen in liquid nitrogen and stored at -120° C until use up to one month later.

Run-on transcription:

RNA elongation reactions were performed for 30 min at 30°C using 2 X 10⁷ nuclei/400 µl reaction. Reaction mixtures were composed of 200 µl nuclei storage buffer plus 200 µl of sterile 2X reaction buffer (10 mM Tris-HCl [pH 8.0], 5 mM MgCl₂, 0.3 M KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 5 mM dithiothreitol, and 2 µl [α-³²P]UTP or [α-³²P]CTP [≈ 3000 Ci/mmol, 10 mCi/ml]). Nucleotides, radionucleotides, and dithiothreitol were added immediately prior to use. Nascent RNA transcripts were allowed to elongate for 30 min at 30°C on a shaking platform, followed by addition of 600 µl of RNase-free DNase I (0.04 units RQ1 DNase I [RNase-free; Promega Corp.], 0.5 M NaCl, 50 mM MgCl₂, 2 mM CaCl₂, 10 mM Tris-HCl [pH 7.4]). The ³²P-labeled RNA was isolated using Trizol (Gibco/BRL), and the final precipitated RNA was dissolved in Church hybridization buffer (1 mM EDTA, 0.5 M NaHPO₄ [pH 7.2], 7% sodium lauryl sulphate [SLS]), to a final concentration of 4 X 10⁶ cpm per ml.

Hybridization of radiolabeled RNA to immobilized unlabeled probes:

In order to distinguish between TS sense and antisense RNA molecules produced in isolated nuclei from transfected cells, target DNA (immobilized on nitrocellulose filters in triplicate dots, 2 µg per dot) consisted of single-stranded synthetic oligonucleotides rather than TS cDNA. Strand-specific oligonucleotide probes were also used to assess levels of human metallothionein-2 (MT-2) mRNA and antisense RNA as positive and negative controls, respectively. Single-stranded oligonucleotides were immobilized on nitrocellulose filters by

dissolving in 6XSSC (16 µg per ml) and applying 125 µl per dot using a BioRad dot-blot apparatus. Unlabeled complementary cDNA probes for GAPDH mRNA (Denhardt *et al.*, 1988) and 18S ribosomal RNA (Behrend *et al.*, 1994) were denatured and immobilized on the same nitrocellulose filters (5 µg per dot, triplicate dots) using a previously-described protocol (Koropatnick, 1988). Hybridization of radiolabeled RNA to these dots assessed transcription of GAPDH and 18S rRNA genes, and acted as internal standards against which to measure changes in TS gene transcription. For cells transfected with single-stranded oligonucleotides, TS gene transcription was assessed by hybridization of radiolabeled TS RNA transcripts to immobilized target DNA consisting of a 1.9 kb *Xho* I fragment isolated from pCHTS-1.

Nitrocellulose filters containing triplicate dots of oligonucleotide and cDNA probes to assess run-on transcription of antisense TS RNA expression vectors, and endogenous TS, MT-2, GAPDH and 18S rRNA genes, were prehybridized in Church buffer for 20 min at 65° C in a Hybaid hybridization chamber. The prehybridization buffer was then removed, 2 mls of radiolabeled RNA resulting from 30 min of run-on transcription in isolated nuclei (in Church hybridization buffer, 4 X 10⁶ cpm per ml) was added, and the filters were hybridized for 48 h at 65° C. The filters were then washed twice at 65°C in posthybridization buffer (40 mM Na₂HPO₄, 1% SDS; 20 min per wash). Posthybridization buffer was removed and 8 ml of RNase A (1 µg per ml in 6XSSC) was added and incubated for 30 min at 37° C to reduce signal from unhybridized radiolabeled RNA. After a final wash in posthybridization buffer (10 min, 37° C) filters were blotted dry and bound radioactivity visualized and quantitated using a phosphorimager and the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). Relative transcription of antisense TS expression vectors, endogenous TS genes, and MT-2 genes was defined as:

$$\text{Relative transcription rate} = \frac{(\text{hybridisation signal from gene of interest})}{(\text{hybridisation signal for GAPDH or 18S rRNA genes})}$$

TS oligonucleotide probes:

Bases in bold-face (below) form part of restriction endonuclease sites, and are not sense or antisense TS sequences. Numbering indicates the distance from the beginning of the transcription start site.

5 ***TS cDNA nucleotides 111 to 140***

sense TS (JK-5): **CTAGATGTGGCCGGCTCGGAGCTGCCGCGCCGGCCA**

antisense TS (JK-2): **AGCTTGGCCGGCGCGGCAGCTCCGAGCCGGCCACAT**

TS cDNA nucleotides 296 to 325

10 *sense TS (JK-3):* **CTAGAGCTACAGCCTGAGAGATGAATTCCCTCTGCA**

antisense TS (JK-4): **AGCTTGCAGAGGGAATTCATCTCTCAGGCTGTAGCT**

MT-2 oligonucleotide probes (Karin and Richards, 1982):

15 Sense and antisense oligonucleotide sequences did not have non-complementary sequences added to the 5' and 3' ends. Numbering indicates the distance from the translation start site.

MT cDNA nucleotides -14 to 6

sense MT: **CTCTTCAGCACGCCATGGAT**

20 ***MT cDNA nucleotides 204 to 223***

antisense MT: **AGGGTCTACCTTTCTTGCGC**

Example 1.2: Antisense oligodeoxynucleotide targeting regions at or near the translation stop site at the 3' end of the TS gene as a method to inhibit growth of human tumour cells

25

a) A 20-mer antisense oligodeoxynucleotide (oligo 86) targeted to the translation stop site at the 3' end of the thymidylate synthase mRNA is growth inhibitory (cytostatic) in a human breast cancer cell line (MCF-7 cells). Antisense oligonucleotides of the same length (oligos 90 and 92), targeted to regions at or near the translation start site at the 5' end of the TS mRNA, are not cytostatic (**Figure 1**).

30

b) A 20-mer antisense oligodeoxynucleotides targeted to the TS mRNA translation start site (oligos 91 and 93) did not inhibit growth of a human cervical carcinoma (HeLa) cell line. In fact, growth was significantly enhanced. Antisense oligodeoxynucleotides targeted to the 3' end of the TS mRNA, including the translation stop site (oligo 86) or a sequence in the 3' untranslated region (oligo 83) significantly inhibited HeLa cell growth. An antisense TS oligonucleotide targeted to another sequence in the 3' untranslated region of TS mRNA (oligo 81) had no effect on HeLa cell growth (**Figures 2 and 3**).

Therefore, no antisense TS oligodeoxynucleotides targeted to the translation start site were successful in inhibiting growth of two different human tumour cell lines (human breast carcinoma MCF-7 cells or human cervical carcinoma HeLa cells). **Two separate antisense TS oligodeoxynucleotides targeted to the 3' end of the TS gene were potent inhibitors of human tumour cell growth.**

Example 1.3:

(a) Antisense oligodeoxynucleotide targeting of the thymidylate synthase translation stop site as a method to enhance human tumour cell sensitivity to the toxic effects of Tomudex (ZD 1694).

Antisense TS oligodeoxynucleotides (oligos 86 and 83) targeting sequences in the 3' untranslated region of TS mRNA enhanced human cervical carcinoma cell sensitivity to Tomudex. The enhancement in sensitivity was in addition to the directly cytostatic effects of oligos 86 and 83 (**Figures 4 and 5**).

(b) Antisense oligodeoxynucleotide targeting of the thymidylate synthase translation start site as a method to enhance human cell resistance to the toxic effects of Tomudex (ZD 1694).

An antisense TS oligodeoxynucleotide (oligo 91) targeting the translation start site of TS mRNA enhanced human cervical carcinoma cell resistance to the toxic effects of Tomudex (Figure 4).

5 **Example 1.4: Induction of transcription of genes targeted with antisense nucleic acids as a screening method to identify appropriate target sequences for antisense nucleic acids.**

Human tumour cells appear to compensate for antisense inactivation of specific mRNA by increasing transcription of genes producing the target sequences, a process that can
10 be termed "compensatory transcription", resulting in resistance to the effectiveness of antisense nucleic acids. It has been observed, that TS gene transcription is induced in human MCF-7 breast carcinoma cells by treatment with antisense TS RNA and oligodeoxynucleotides targeted to regions at or near the TS mRNA translation start site. The same phenomenon has been observed in human HeLa cells transiently-transfected with
15 antisense TS oligo 91 (targeted to the translation start site), but not in response to oligo 86 (targeted to the translation stop site) (Figure 6). Increased specific gene transcription in response to transfected antisense nucleic acids would indicate that the target sequence is inappropriate to achieve downregulated gene expression. On the other hand, it may be an appropriate sequence to target to achieve upregulated gene expression (to increase resistance
20 to chemotherapeutic drugs, for example, in normal tissues.

In summary, the present invention has demonstrated that antisense oligonucleotides, targeted against selected regions of thymidylate synthase mRNA, can effectively inhibit growth when administered alone. They can also enhance cell killing by Tomudex. Conversely,
25 antisense oligonucleotides targeted to certain mRNA regions (for example, the translation start site) may either be ineffective, or enhance growth and survival during exposure to Tomudex. Ineffectiveness may be due to oligonucleotide-induced TS gene transcription. It is essential to identify TS mRNA regions that may be effectively targeted with antisense sequences to inhibit tumour cell growth and enhance the toxicity of anticancer drugs. Furthermore, the mechanism
30 by which antisense sequences targeted to 5' TS mRNA regions induce TS gene transcription has

important implications for choosing antisense targets in TS mRNA in particular, and for optimising antisense strategies in general.

Example 2

Example 2.1: Experimental Methods

Oligonucleotides:

Fully phosphorothioated 20-base oligonucleotides were synthesised by ISIS Pharmaceuticals (Carlsbad, California, USA). The 6 nucleotides on either end of the oligomer were methoxyethoxylated in the 2'-position, enhancing hybridisation as well as resistance to exonucleation. The middle 8 nucleotides were not methoxyethoxylated to allow RNase H endonucleation and degradation of mRNA hybridised to the oligomer. ODN 83 is complementary to TS mRNA, starting from a position 136 bases downstream of the translational stop site (5'-GCCAGTGGCAACATCCTTAA-3'). ODN 32 is a randomised sequence of ODN 83 (5'-ATGCGCCAACGGTTCCTAAA-3'), with the same base constituents in random order. A search of available mRNA sequences using the NCBI BLAST search tool revealed that ODN 83 had sequences of 10 or more complementary bases to only human TS mRNA, while ODN 32 had sequences of 10 or more complementary bases to no known mRNAs.

Radioisotope:

[6-³H]5-FUdR (specific activity 18.6 Ci/mmol) was purchased from Moravek Biochemicals (Brea, California, USA). This isotope was 99.98% pure upon initial production, with a degradation rate of 0.5-1% per month at -20°C, and was used within 3 months of manufacture.

Other supplies:

Cell culture chemicals and nutrients were obtained from Canadian Life Technologies (GIBCO) (Burlington, Ontario, Canada). All other chemicals were obtained from commercial

sources. Plasticware was purchased from VWR Canlab (Mississauga, Ontario, Canada) and Fisher Scientific Uniondale, Ontario, Canada).

Cell Culture:

Human cervical carcinoma HeLa cells were maintained in D-MEM plus 10% foetal bovine serum and penicillin (50 units/ml)/streptomycin (50 µg/ml). Cultures were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Rapidly proliferating cells were utilised for establishing cultures of experimental cells, which were allowed to plate overnight prior to manipulation.

Transfection was performed using lipofectamine (LFA, GIBCO-BRL), a polycationic liposome formulation. Cells to be used for proliferation experiments were plated at a starting cell number of between 0.6 and 1 x 10⁵ cells per 25-cm tissue culture flask, and LFA was used at 3 µg/ml. For cells in 75-cm flasks, which were to be harvested and extracted for assay of mRNA or TS content, the starting cell number was approximately 8 - 10 x 10⁵, and the LFA concentration was 4 µg/ml. Prior to transfection, adherent HeLa cells were washed once with PBS and then treated with antisense or scrambled control ODN (50 nM) in the appropriate concentration of LFA in serum-free D-MEM, at 37°C for 6.0 h. The cells were then washed once with PBS and cultured in the presence of D-MEM plus 10% FBS. In cells treated with cytotoxic agents, exposure was initiated 24 hours after the removal of LFA/ODN, by addition of 0.2-volume of growth medium containing the agent at 6 times the final concentration. At the time of addition of drug, and after 4 days of incubation, cell numbers were determined from replicate flasks by enumerating with a particle counter (Coulter Electronics, Hialeah, Florida, USA). The proliferation of drug treated cells (fold-increase in cell number) was calculated as a percentage of that of the control cells. IC₅₀ and IC₉₀ values were determined by interpolation of plotted data.

RT-PCR to measure TS mRNA:

RNA was isolated from transfected cells using Trizol (GIBCO-BRL). Complementary DNA was synthesised from 1 µg of total RNA using 200 U of Moloney Murine Leukemia Virus reverse transcriptase (GIBCO BRL) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 1 mM mixed dNTPs, 100 pmol random primers and 10 mM dithiothreitol at 37°C for 1

hr. The enzyme was inactivated at 95°C for 5 min. The resulting cDNAs (in a volume of 2.5 µl) were amplified in a polymerase chain reaction (PCR) using 1.25 U of *Taq* DNA polymerase in 50 µl of 20 mM Tris-HCl pH 8.4), 50 mM KCl, 0.2 mM mixed dNTPs, 2 mM MgCl₂, and 50 pmol of primers specific for TS and GAPDH cDNAs. TS and GAPDH cDNAs were amplified together in the same reaction tube to allow the level of housekeeping GAPDH cDNA to be used to determine the relative level of TS mRNA. Twenty-four to 27 cycles of PCR amplification (94°C 45 s, 55°C 30 s, 72°C 90 s) produced fragments of 208 bp and 752 bp using primer sets for TS (forward 5'CACACTTTGGGAGATGCACA3'; reverse 5'CTTTGAAAGCACCTAAACAOCAT3') and GAPDH (forward 5'TATTGGGCGCCTGGTCACCA3'; reverse 5'CCACCTTCTTGATGTCATCA3'), respectively. PCR products were separated on a 1.2% agarose gel, and transferred to Hybond nylon membrane (Amersham, Canada, Ltd., Oakville, Ontario, Canada) by Southern blotting. Blots were hybridised to [α -³²P]dCTP random primer-labeled probe (pcHTS-1, a generous gift from Dr. K. Takeishi, University of Shizuoka, Shizuoka, Japan; or a cDNA insert recognising glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). Hybridisation signals were quantified using a PhosphorImager and ImageQuant (Molecular Dynamics, Sunnyvale, California, USA).

TS binding assay:

Cellular content of TS was assayed by binding of [6-³H]5-FdUMP. This method was demonstrated to label total TS unless the cells were pretreated with 5-FU or 5-FUdR. The assay was performed using cells that were treated with antisense ODN 83 or the scrambled control ODN 82. Briefly, cells were harvested by scraping into PBS and resuspending the subsequent pellet in 100 mM KH₂PO₄ (pH 7.4). Cells were disrupted by freezing and thawing, followed by sonication. The total protein concentration was determined using Coomassie staining (BioRad reagent) (MI) in order to express results as pmol 5-FdUMP bound per mg total protein. 5-FdUMP binding was assessed in paired lysates from cells transfected with ODN 83 or ODN 32, in separate incubation reactions carried out on different days; however, pairs were always assessed together under the same reaction conditions. On each occasion, the incubation vessel contained 50 µg of total protein, 75 µM methylene-FH₄, 100 mM mercaptoethanol, 50 mM KH₂P0₄ (pH 7.4), and 15 nM [6-³H]5-FdUMP in a final

volume of 200 μ l. After 30 min at 37°C, the incubation was stopped by addition of 5 volumes of albumin-coated, activated charcoal. After 10 min (room temperature), this slurry was centrifuged (3000 x g, 30 min, 22°C), and the supernatant re-centrifuged to completely remove particulate matter. Two aliquots of 300 μ l each were removed from the final, clarified supernatant for scintillation counting.

Statistical analysis:

Data for cell growth after treatment with ODNs alone, or in combination with cytotoxic drugs, are presented as the mean \pm standard error or standard deviation as determined by Student *t*-test. For determinations of FdUMP binding, differences between paired samples from cells transfected with different ODNs were assessed using a paired *t*-test. This controlled for differences in experimental conditions on each of the 5 occasions that FdUMP binding was assessed. In all cases, significance was chosen *a priori* to be indicated by differences at a confidence level of $p < 0.02$.

References

- Almendral, J.M., *et al.* Mol. Cell. Biol. 8:2140-2148, 1988.
- Ayusawa, D., *et al.* J. Mol. Biol. 190:559-567, 1986.
- Behrend, E.I., *et al.* Cancer Res. 54:832-837, 1994.
- Calvert, A.H., *et al.* J. Clin. Oncol. 4:1245-1252, 1986.
- Chu, E., *et al.* Proc. Natl. Acad. Sci. USA 88:8977-8981, 1991.
- Chu, E., *et al.* Mol. Pharm. 43:527-533, 1993a.
- Chu, E., *et al.* Mol. Cell. Biol. 15:179-185, 1995.
- Chu, E., *et al.* Mol. Cell. Biol. 14:207-213, 1994.
- Chu, E., *et al.* Proc. Natl. Acad. Sci. USA 90:517-521, 1993b.
- Chu, E., *et al.* Cancer Res. 50:5834-5840, 1990.
- Church, G.M., *et al.* Proc. Natl. Acad. Sci. USA 81:1991-1995, 1984.
- Danenberg, P.V., Biochim. Biophys. Acta 473:73-92, 1977.
- DeGregori, J., *et al.* Mol. Cell. Biol. 15:4215-4224, 1995.
- Denhardt, D.T., *et al.* Oncogene 2:55-59, 1988.
- Farnham, P.J., *et al.* Biochim. Biophys. Acta Rev. Cancer 1155:125-131, 1993.

- Hardy, L.W., *et al.* Science 235:448-455, 1987.
- Heidelberger, C., *et al.* Adv. Enzymol. 54:58-119, 1983.
- Jackman, A.L., *et al.* Adv. Enzyme Regul. 31:13-27, 1991a.
- Jackman, A.L., *et al.* Cancer Res. 51:5579-5586, 1991b.
- 5 Jelinek, W.R., *et al.* Proc. Natl. Acad. Sci. USA 77:1398-1402, 1980.
- Jenh, C.-H., *et al.* Mol. Cell. Biol. 5:2527-2532, 1985.
- Johnson, L.F. *et al.* J. Cell. Biochem. 54:387-392, 1994.
- Karin, M., *et al.* Nucleic Acids Res. 10:3165-3173, 1982.
- Keyomarsi, K., *et al.* J. Biol. Chem. 268:15142-15149, 1993.
- 10 Kikuchi, K., *et al.* J. Biol. Chem. 267:21505-21511, 1992.
- Koropatnick, J., *et al.* Proc. Soc. Exp. Biol. Med. 188:287-300, 1988.
- Koropatnick, J., *et al.* BioTechniques 22:64-66, 1997.
- Koropatnick, J., *et al.* Mol. Biol. Med. 5:69-83, 1988.
- Lochshin, A., Proc. Natl. Acad. Sci. USA 76:750-754, 1979.
- 15 Maley, F., *et al.* J. Biol. Chem. 235:2968-2970, 1960.
- Marzluff, W.F., *et al.* IRL Press, Oxford, 89-129, 1984.
- McKay, R.A., *et al.* Nucleic Acids Res. 24:411-417, 1996.
- Mudrak, I., *et al.* Mol. Cell. Biol. 14:1886-1892, 1994.
- Navalgund, L.G., *et al.* J. Biol. Chem. 255:7386-7390, 1980.
- 20 Peters, G.J., *et al.* Cancer Res. 46:20-28, 1986.
- Peters, G.J., *et al.* Eur. J. Cancer 30A:1408-1411, 1994.
- Rapaport, E., *et al.* Proc. Natl Acad. Sci. USA 89:8577-8580, 1992.
- Sambrook, J., *et al.* T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
- 25 Suzuki, M., *et al.* Oncology 51:334-338, 1994.
- Takeishi, K., *et al.* Nucleic Acids Res. 13:2035-2043, 1985.
- Voeller, D.M., *et al.* Nucleic Acids Res. 23:869-875, 1995.
- Volm, M., *et al.* Anticancer Res. 12:2293-2296, 1992.
- Volm, M., *et al.* Anticancer Res. 14:1271-1276, 1994.

CLAIMS

1. An antisense oligonucleotide which hybridises to a target nucleic acid sequence in thymidylate synthase and which selectively inhibits thymidylate synthase production in mammalian cells.
2. An antisense oligonucleotide which hybridises to a target nucleic acid sequence in thymidylate synthase and which selectively enhances thymidylate synthase production in mammalian cells.
3. The antisense oligonucleotide of claim 1 or claim 2 comprising SEQ ID No 1, 2, 3, 4, 5, 6, or 7.
4. The antisense oligonucleotide of claim 1 or claim 2 comprising SEQ ID No 8 or 9.
5. A combination product comprising an antisense oligonucleotide targeted to thymidylate synthase in combination with an anticancer agent.
6. The combination product of claim 5 in which the anticancer agent is a thymidylate synthase inhibitor or a cytostatic agent or an antiproliferative drug.
7. The combination product of claim 5 in which the anticancer agent is Tomudex or ZD9331.
8. A pharmaceutical composition comprising the combination product of claim 5 and a pharmaceutically acceptable diluent or carrier.
9. A method for the treatment of cancer or for providing an antiproliferative effect which comprises administering to a warm-blooded animal an effective amount of the combination product claimed in claim 4.

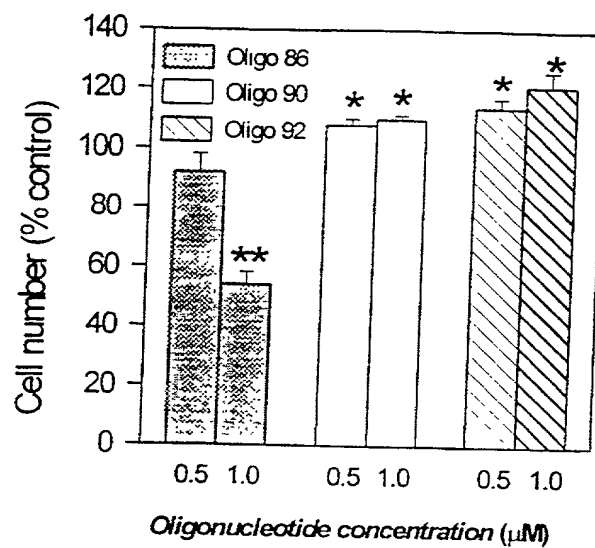
-29-

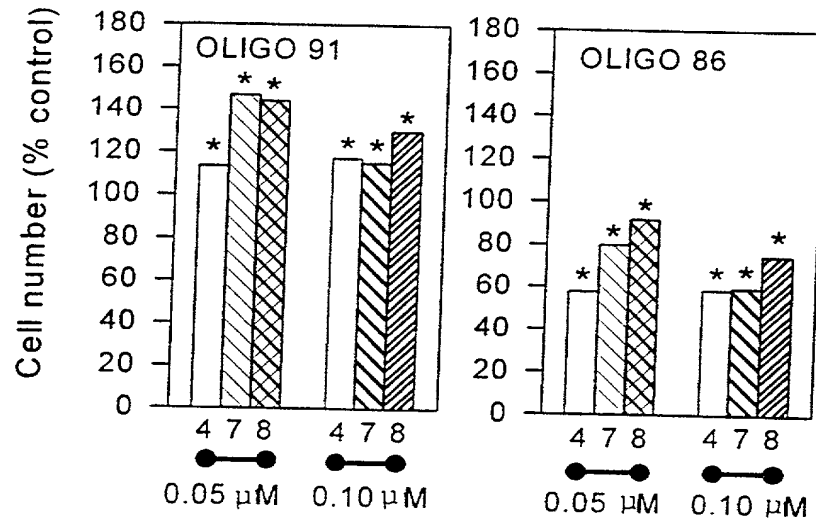
10. Use of the combination product claimed in claim 4 in the production of a new medicament for the treatment of cancer or for treatment of antiproliferative disease.

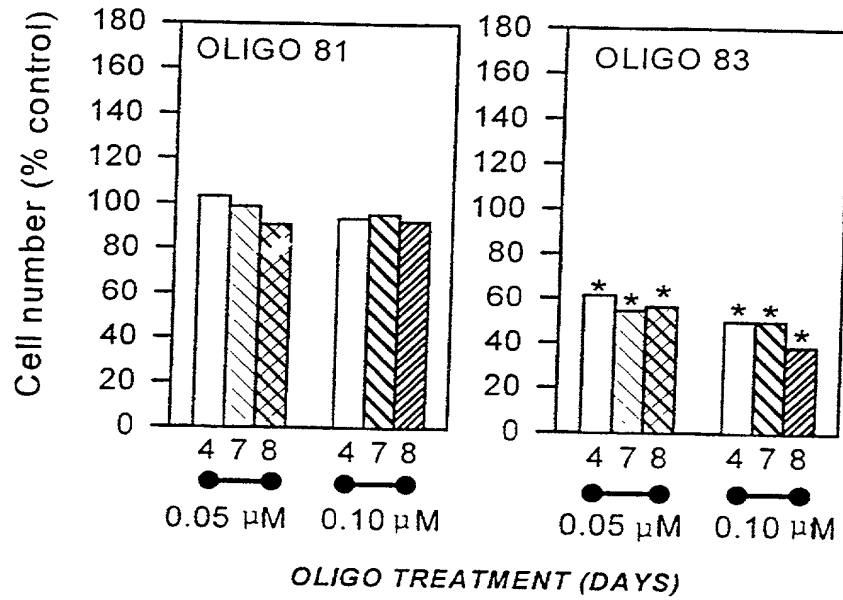
5

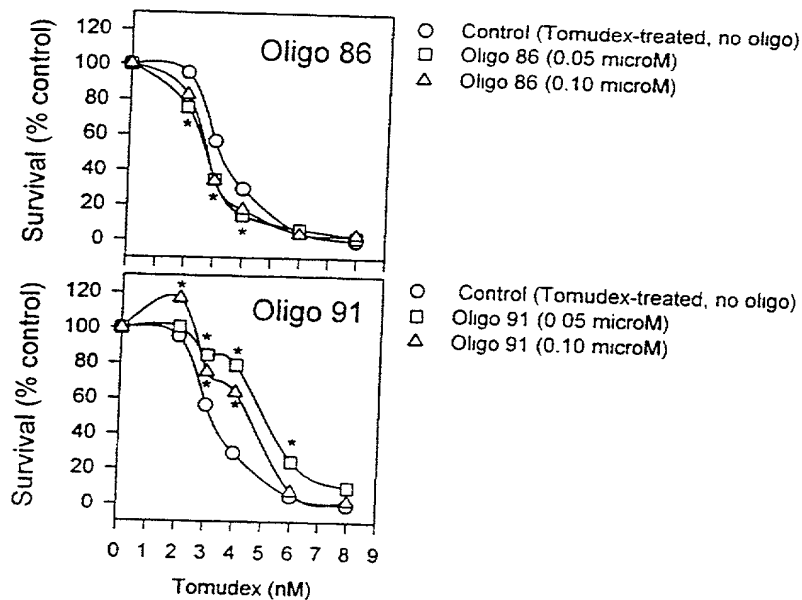
0011208760560

1/13

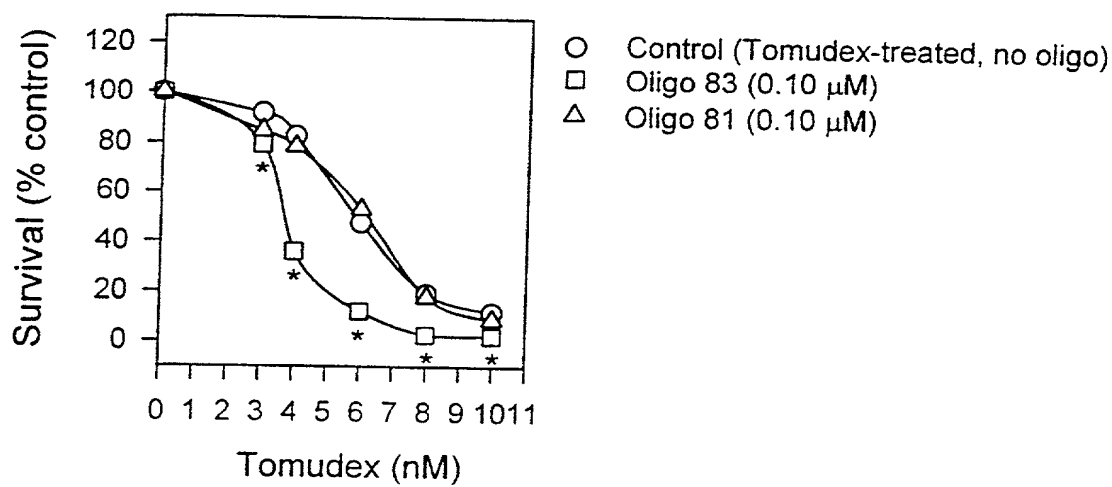
**Figure 1**

**Figure 2**

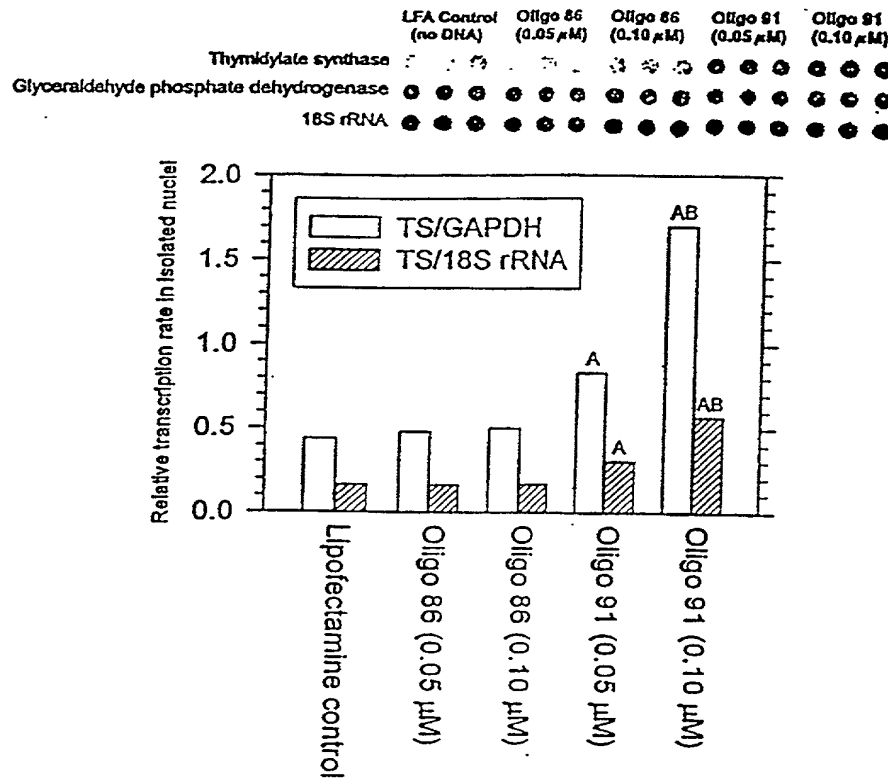
**Figure 3**

**Figure 4**

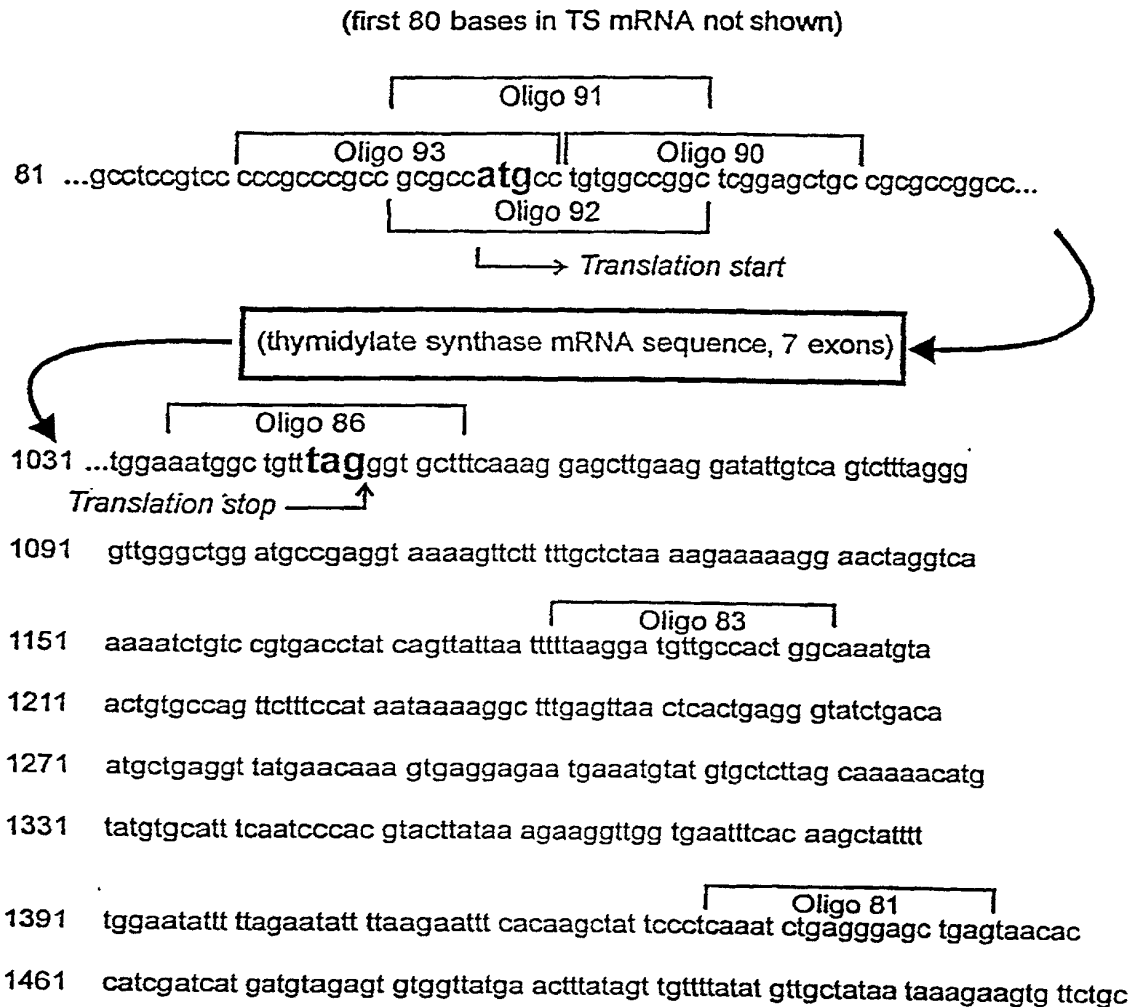
5/13

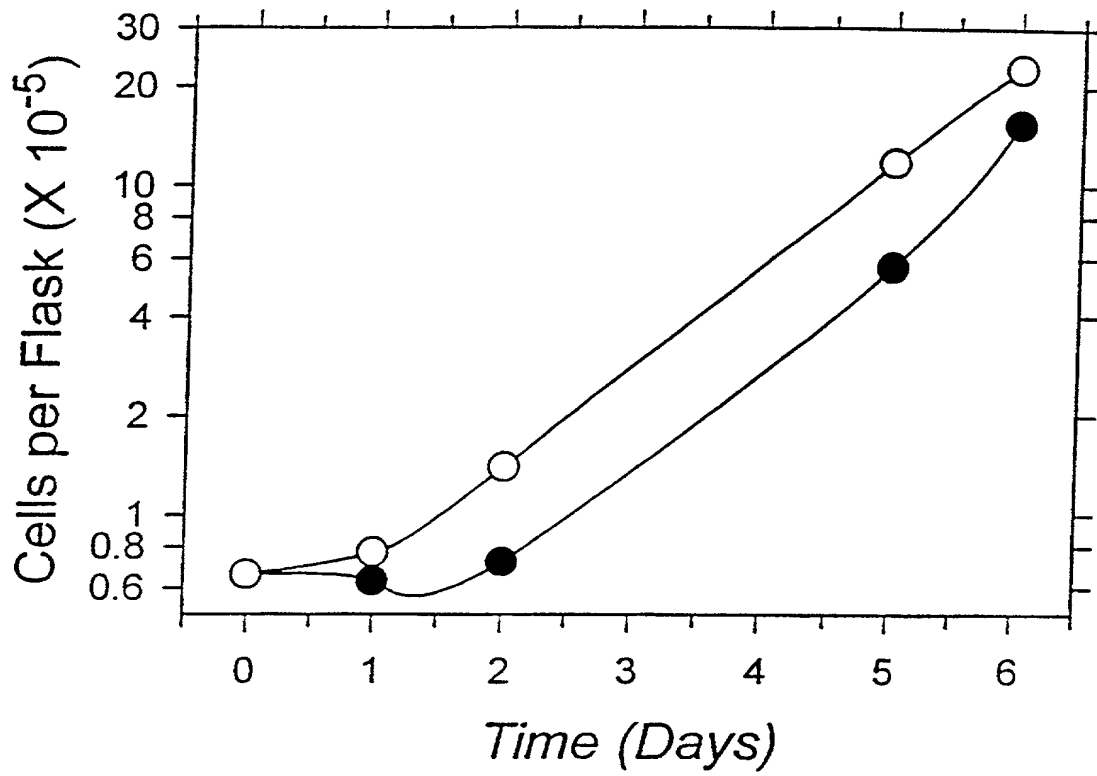
**Figure 5**

6/13

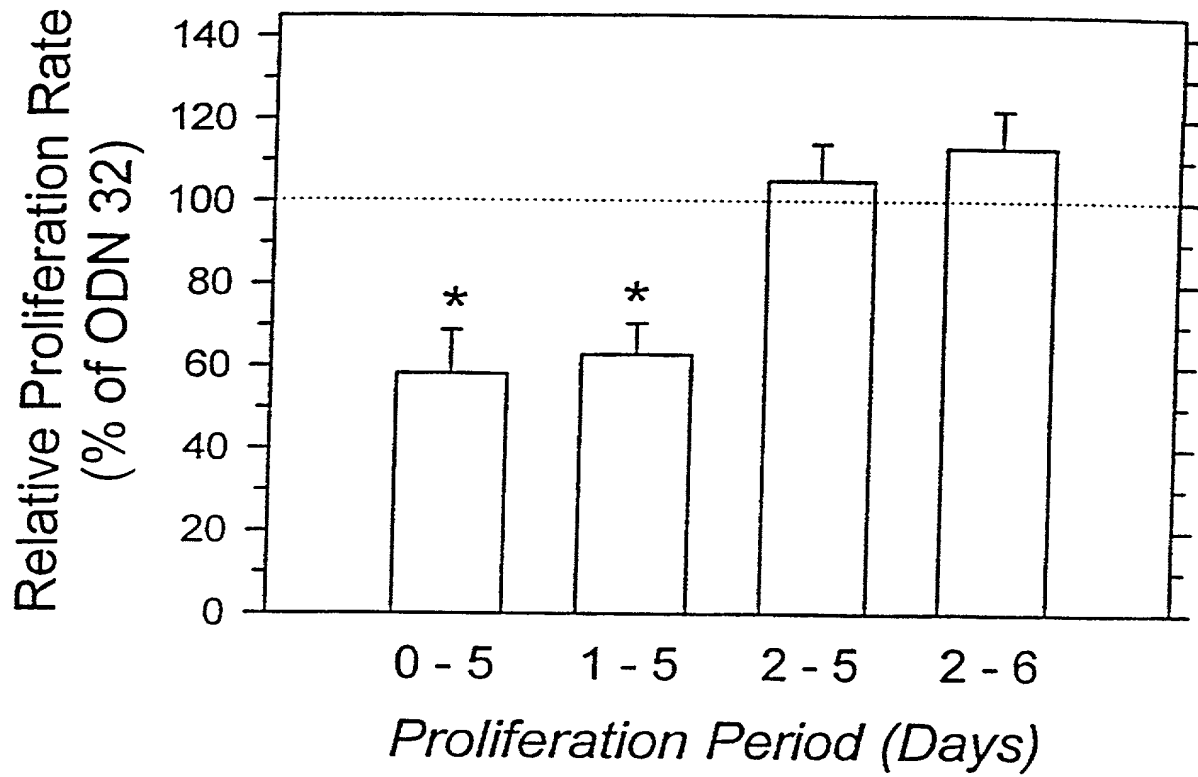
**Figure 6**

7/13

**Figure 7**

FIGURE 8

9/13

FIGURE 9

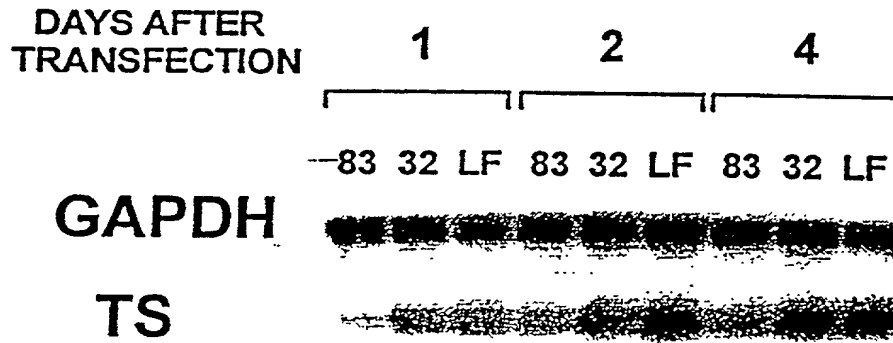
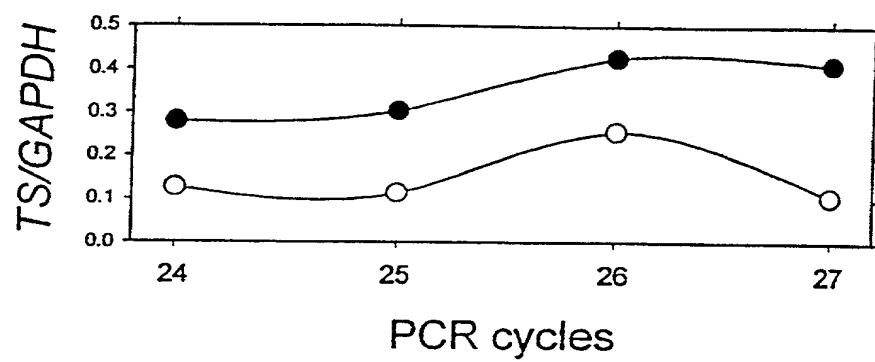


FIGURE 10

FIGURE 11

12/13

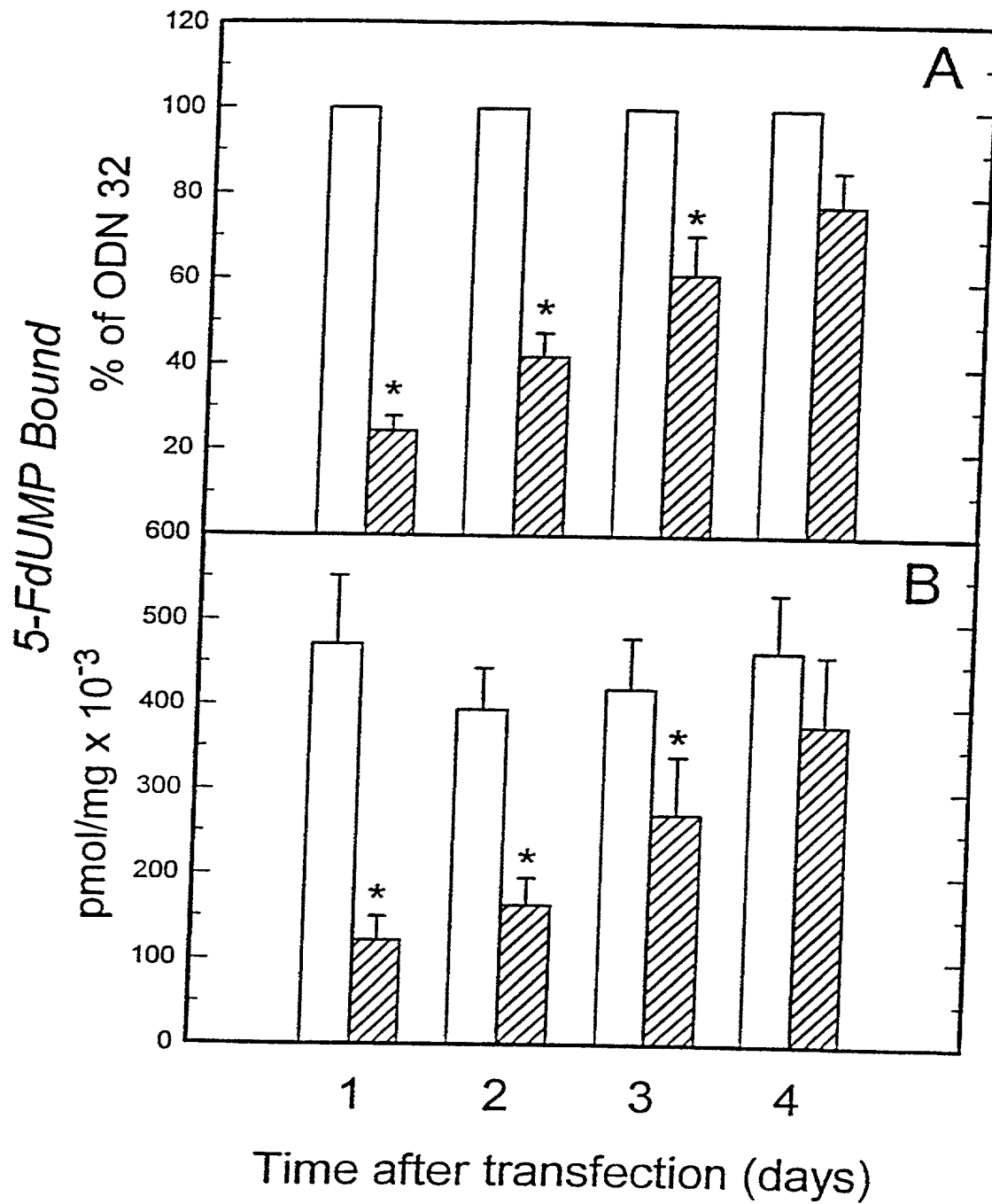
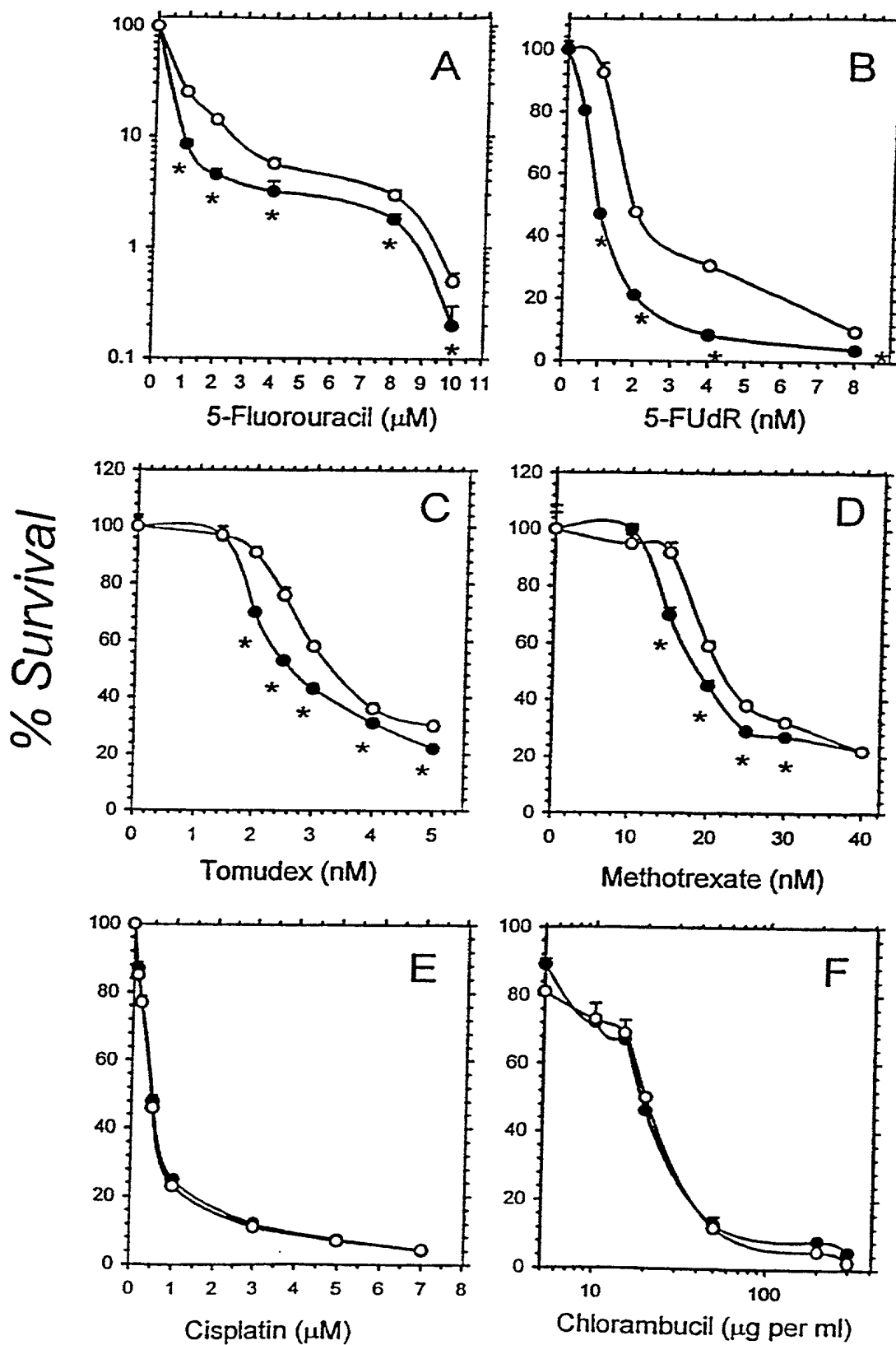
FIGURE 12

FIGURE 13



FOR UTILITY/DESIGN
CIP/PCT NATIONAL/PLANT
ORIGINAL/SUBSTITUTE/SUPPLEMENTAL
DECLARATIONS

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PHM. 70275/UST

PM & S
FORM

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the INVENTION ENTITLED
ANTISENSE OLIGONUCLEOTIDES AGAINST THYMIDYLATE SYNTHASE

the specification of which (CHECK applicable BOX(ES))

X
BOX(ES) → A. ☐ is attached hereto.
→ B. ☐ was filed on _____ as U.S. Application No. _____ /
→ C. ☒ was filed as PCT International Application No. PCT/ GB98/02820 on 17 September 1998
and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. Except as noted below, I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International Application which designated at least one other country than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International Application, filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application:

<u>PRIOR FOREIGN APPLICATION(S)</u> <u>Number</u>	<u>Country</u>	<u>Day/MONTH/Year Filed</u>	<u>Date first Laid-</u> <u>open or Published</u>	<u>Date Patented</u> <u>or Granted</u>	<u>Priority NOT Claimed</u>
9720107.3	United Kingdom	23 Sep 1997			
9722012.3	United Kingdom	17 Oct 1997			
9812140.3	United Kingdom	6 Jun 1998			

If more prior foreign applications, X box at bottom and continue on attached page.

Except as noted below, I hereby claim domestic priority benefit under 35 U.S.C. 119(e) or 120 and/or 365(c) of the indicated United States applications listed below and PCT international applications listed above or below and, if this is a continuation-in-part (CIP) application, insofar as the subject matter disclosed and claimed in this application is in addition to that disclosed in such prior applications, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of each such prior application and the national or PCT international filing date of this application:

<u>PRIOR U.S. PROVISIONAL, NONPROVISIONAL AND/OR PCT APPLICATION(S)</u> <u>Application No. (series code/serial no.)</u>	<u>Day/MONTH/Year Filed</u>	<u>Status</u> <u>pending, abandoned, patented</u>	<u>Priority NOT Claimed</u>
--	-----------------------------	--	-----------------------------

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Pillsbury Madison & Sutro LLP, Intellectual Property Group, 1100 New York Avenue, N.W., Ninth Floor, East Tower, Washington, D.C. 20005-3918, telephone number (202) 861-3000 (to whom all communications are to be directed), and the below-named persons (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to delete names/numbers below of persons no longer with their firm and to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct the above firm and/or a below attorney in writing to the contrary.

Paul N. Kokulis	16773	Dale S. Lazar	28872	Mark G. Paulson	30793	Michael R. Dzwonczyk	36787
Raymond F. Lippitt	17519	Paul E. White, Jr.	32011	Stephen C. Glazier	31361	W. Patrick Bengtsson	32456
G. Lloyd Knight	17698	Glenn J. Perry	28458	Paul F. McQuade	31542	Jack S. Barufka	37087
Carl G. Love	18781	Kendrew H. Colton	30368	Ruth N. Morduch	31044	Adam R. Hess	41835
Kevin E. Joyce	20508	G. Paul Edgell	24238	Richard H. Zaitlen	27248		
George M. Sirilla	18221	Lynn E. Eccleston	35861	Roger R. Wise	31204		
Donald J. Bird	25323	Timothy J. Klima	34852	Jay M. Finkelstein	21082		
Peter W. Gowdey	25872	David A. Jakopin	32995	Anita M. Kirkpatrick	32617		

(1) INVENTOR'S SIGNATURE: Donald James Koropatnick Date: 9th May, 2000
First Middle Initial Family Name
Residence City State/Foreign Country Country of Citizenship
Post Office Address (include Zip Code)
790 Commissioners Road East, London, Ontario N6A 4L6, Canada

(2) INVENTOR'S SIGNATURE: Mark Vincent Date: 9th May, 2000
First Middle Initial Family Name
Residence City State/Foreign Country Country of Citizenship
Post Office Address (include Zip Code)
790 Commissioners Road East, London, Ontario N6A 4L6, Canada

FOR ADDITIONAL INVENTORS, "X" box ☒ and proceed on the attached page to list each additional inventor.
☒ See additional foreign priorities on attached page (incorporated herein by reference).

Atty. Dkt. No. PM

(M#)

DECLARATION AND POWER OF ATTORNEY

(continued)

ADDITIONAL INVENTORS

(3) INVENTOR'S SIGNATURE: *Mark*Date: X 17th FEBRUARY 2000

NICHOLAS		M	DEAN
First		Middle Initial	Family Name
Residence	Carlsbad	California	U.S.A.
City		State/Foreign Country	Country of Citizenship
Post Office Address	2292 Faraday Avenue, Carlsbad, CA 92008, U.S.A.		
(include Zip Code)			

(4) INVENTOR'S SIGNATURE:

Date:

First		Middle Initial	Family Name
Residence			
City		State/Foreign Country	Country of Citizenship
Post Office Address			
(include Zip Code)			

(5) INVENTOR'S SIGNATURE:

Date:

First		Middle Initial	Family Name
Residence			
City		State/Foreign Country	Country of Citizenship
Post Office Address			
(include Zip Code)			

(6) INVENTOR'S SIGNATURE:

Date:

First		Middle Initial	Family Name
Residence			
City		State/Foreign Country	Country of Citizenship
Post Office Address			
(include Zip Code)			

(7) INVENTOR'S SIGNATURE:

Date:

First		Middle Initial	Family Name
Residence			
City		State/Foreign Country	Country of Citizenship
Post Office Address			
(include Zip Code)			

(8) INVENTOR'S SIGNATURE:

Date:

First		Middle Initial	Family Name
Residence			
City		State/Foreign Country	Country of Citizenship
Post Office Address			
(include Zip Code)			

(9) INVENTOR'S SIGNATURE:

Date:

First		Middle Initial	Family Name
Residence			
City		State/Foreign Country	Country of Citizenship
Post Office Address			
(include Zip Code)			